

AMENDMENTS TO THE SPECIFICATION

Please amend the specification as follows:

Please replace the Title with the following amended Title:

Polypeptides, Compositions, and Methods ~~methods~~ for cleaving IAP

Please cancel the Sequence Listing as filed in the original application.

Please enter the substitute Sequence Listing set forth in Exhibit A on the next page (pg. 96) after the claims.

Please replace the paragraph numbered [0011] with the following amended paragraph:

[0011] Smac/DIABLO and Omi/HtrA2 are two molecules identified as antagonists of IAPs. These molecules can reactivate the IAP-inhibited caspases. Smac and Omi are nuclear-encoded mitochondria proteins. It is known that after being synthesized in the cytoplasm, Smac and Omi are quickly imported into the mitochondria by the N-terminal mitochondria targeting peptides. The cleavage of peptides attached to Smac or Omi, inside the mitochondria, generates active Smac and Omi molecules with a new apoptogenic N-terminus, named the IAP binding motif. This motif consists of a short stretch of hydrophobic amino acids AVPI (SEQ ID NO. 84) and AVPS (SEQ ID NO. 77) in Smac and Omi, respectively. It has been observed that in the cytosol, the IAP binding motifs of Smac and Omi antagonize IAPs' inhibition of caspases by competitively binding to the BIR2 and BIR3 domains of IAPs so the BIR domain-bound caspases

are released and reactivated. As such, it has been determined that Smac and Omi competitively bind LAP to prevent IAP inhibition of caspase.

Please replace the paragraph numbered [0030] with the following amended paragraph:

[0030] FIG. 1(A-E) relate ~~relates~~ to Omi cleavage of IAP proteins, different IAP proteins were incubated in the absence or presence of various amounts of Omi WT or the protease dead mutant Omi SA;

Please replace the paragraph numbered [0036] with the following amended paragraph:

[0036] FIG. 2(A-D) show ~~shows~~ Omi/HtrA2 Cleavage of cIAP1 and the relation to the AVPS IAP binding motif;

Please replace the paragraph numbered [0041] with the following amended paragraph:

[0041] FIG. 3(A-B) show ~~shows~~ cIAP1 cleavage by Omi/HtrA2 and how cleavage reduces cIAP 1's caspase inhibitory activity;

Please replace the paragraph numbered [0044] with the following amended paragraph:

[0044] FIG. 4(A-B) show ~~shows~~ that cIAP1 cleavage by Omi/HtrA2 attenuates its Ub ligase activity on caspase substrates;

Please replace the paragraph numbered [0047] with the following amended paragraph:

[0047] FIG. 5(A-B) show ~~shows~~ mapping of Omi/HtrA2 cleavage sites on clAP1;

Please replace the paragraph numbered [0049] with the following amended paragraph:

[0049] FIG. 5B shows a map of Omi cleavage sites on human clAP1, the clAP1 is labeled with the three mapped and unmapped sites, the three underlined amino acid sequences were the amino terminal sequences (determined by Edman Degradation) of the cleaved clAP1 fragments F1/F2, F3 and F4, respectively, Omi cleaves clAP1 (SEQ ID NO. 78) after the residue Thr4, Asn133, and Leu161 as denoted by the three arrows, both polypeptide fragments F1 and F2 start with the amino acid sequence ASQRLFPG (SEQ ID NO. 85), F4 starts ~~F6 starts~~ with SFAHSLSP (SEQ ID NO. 86), and F3 ~~F5~~ with NSRAVEDI (SEQ ID NO. 87);

Please replace the paragraph numbered [0050] with the following amended paragraph:

[0050] FIG. 6(A-D) show ~~shows~~ that Omi cleaves clAP1 in cells, and this cleavage promotes caspase activation in etoposide-induced cell death;

Please replace the paragraph numbered [0063] with the following amended paragraph:

[0063] As mentioned, the Omi WT nucleic acid sequence includes three codons which, when expressed, form a catalytic triad in the protease molecule. The nucleic acids, which form the codons, are at positions 193-195, 283-285, and

517-519 on the mature Omi WT nucleic acid sequence, SEQ ID NO. 1. The PDZ domain is located between nucleic acids 675 and 975 of SEQ ID NO. 1. The AVPS nucleic acids are nucleotides 1-12 of SEQ ID NO. 1. The hinge sequence is located between nucleic acids 636 and 675. As will be shown, these sequences can be removed or mutated. It is important to note that the Omi WT nucleic acid sequence should encode a serine at position 306 (S306) of the active protease polypeptide expressed by the wild-type version of Omi. Thus, the expressed polypeptide includes a catalytic triad, which includes S306. The mature polypeptide, SEQ ID NO. 44, shows the serine at position 173. This is a mature Omi polypeptide that excludes 133 amino acid residues located prior to the AVPS sequence (SEQ ID NO. 77). As such, S306 and S 173 are interchangeable for purposes of this application. Either way, the Omi polypeptide enzymatically cleaves IAP.

Please replace the paragraph numbered [0187] with the following amended paragraph:

[0187] To examine whether the differential in catalytic efficiency between Omi WT and Omi Δ 8 was due to different binding affinities to IAPs, a GST-based pulldown assay was used to monitor the IAP binding affinities of Omi WT and Omi Δ 8. Full-length cIAP1 (50 nM) was incubated with 100 nM of Omi WT and Omi mutants for 20 minutes at 4° C. in 50 μ l of PBST. The samples were then incubated with 20 μ l of Glutathione SEPHAROSE® (agarose) Sepharose beads (Amersham Biosciences, Piscataway, N.J.) for 30 minutes at 4° C. The beads

were precipitated by centrifugation and washed briefly with 1.4 mlx3 of PBST. The proteins bound to the beads and left in the supernatant were separately mixed with SDS sample loading buffer and resolved by SDS-PAGE, then transferred to a nitrocellulose filter. The upper part of the filter was probed with an antibody against GST and the lower part with an antibody against Penta-His. As shown in FIG. 2C, Omi WT could bind to a cIAP1, whereas Omi Δ 8 completely lost IAP binding, which correlated with the catalytic activity. This indicated that the direct binding of Omi to IAPs was required for Omi to efficiently cleave IAPs, and this IAP binding motif-directed association between Omi and cIAP1 greatly accelerated the proteolytic efficiency for cIAP1.

Please replace the paragraph numbered [0192] with the following amended paragraph:

[0192] Since Omi and Smac bind to IAP proteins through the N-terminal conserved tetrapeptide AVPS (SEQ ID NO. 77) and AVPI (SEQ ID NO. 84), respectively, it is reasonable to speculate that there are some functional interactions between Omi and Smac in the context of IAP cleavage. This was analyzed first by determining if Omi could proteolytically process Smac. Not surprisingly, when incubated with Omi, either in the presence or absence of cIAP1, Smac stayed in its unprocessed form throughout the reaction. In an immunoprecipitation assay, Smac and Omi did not bind to each other regardless of the presence of cIAP1. This result excluded the possibility of a direct interaction between the two molecules. Smac, therefore, was not cleavable by Omi. The SDS-PAGE and Western Blot data is not shown.

Please replace the paragraph numbered [0216] with the following amended paragraph:

[0216] Expression and purification of recombinant proteins from bacteria was accomplished using the following protocol. The C-terminal hexa-His tagged wild-type and mutant Omi proteins were over-expressed in *E. coli* strain B121 (DE3) and purified with Ni-NTA SEPHAROSE[®] (agarose) ~~Sepharese~~ (QIAGEN, Valencia, Calif.) affinity chromatography. The N-terminal hexa-His tagged Livin α and Livin β were expressed in *E. coli* strain JM109, and the recombinant proteins were purified with Ni-NTA SEPHAROSE[®] (agarose) ~~Sepharese~~ affinity chromatography and further fractionated with Q-SEPHAROSE[®] (agarose) ~~Q-Sepharese~~ ion exchange chromatography (Amersham). The GST-fusion forms of cIAP1, cIAP2, XIAP, and DIAP1 were expressed in *E. coli* strain B121 (DE3) and purified with Glutathione Q-SEPHAROSE[®] (agarose) ~~Sepharese~~ affinity chromatography followed by Superdex[™] (dextran/cross-linked agarose) ~~Superdex~~ 200 gel filtration chromatography (Amersham). The purity of proteins was checked by SDS-PAGE. The protein concentrations were determined by the modified Bradford method (Zor and Selinger, 1996).

Please replace the paragraph numbered [0218] with the following amended paragraph:

[0218] The assay for caspase inhibitory activity of cIAP1 was as follows. In vitro translated, ³⁵S-labeled, procaspase-3 (1.5 μ l) was mixed in a reaction volume for 40 minutes at 30° C. The reaction was carried out in the presence or absence

of different IAP proteins and/or various forms of Omi protein. The reaction was stopped with the addition of 7 μ l of 4xSDS sample loading buffer and the samples were separated on 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a phosphor screen (Amersham) for one hour at room temperature. The in vitro transcription and translation of 35 S-labeled procaspase-3 was carried out with the T_NT T7 coupled reticulocyte lysate system from Promega (Madison, Wis.). This His-tagged procaspase-3 was further purified with Ni-NTA SEPHAROSE[®] (agarose) Sepharose column before using.

Please replace the paragraph numbered [0233] with the following amended paragraph:

[0233] Finally, Fab-Omi liposomes utilizing biotinylated Fab molecules, biotinylated liposomes and avidin are prepared. The biotinylated Fab fragments in PBS are mixed with a twenty-fold molar excess of egg white avidin (Vector Labs, Burlingame, Calif.; Sigma Chemical, St. Louis, Mo.), incubated overnight at 4° C. The excess avidin is removed by passage of the mixture over anti-human light chain affinity columns (e.g., Pharmacia SEPHAROSE[®] (agarose) Sepharose 4B). Fab-biotin-avidin molecules are eluted with citrate buffer, pH of then pooled fractions are dialyzed against PBS, pH=7.0. A suspension of biotinylated Omi mutant protein (Omi)-containing liposomes is mixed with Fab-biotin-avidin solutions in PBS to yield avidin to free biotin ratios on the liposome surfaces of approximately 2:1, 5:1, 10:1, and 20:1 molar ratios. After incubation overnight at 4° C. on a rotational shaker, liposomes are passed through a Pharmacia

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Sephadex G200 column. The Fab-Omi liposomes are collected in the void volume and resuspended in PBS. The resultant Fab-Omi liposomes are available for use.